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A Low Phytic Acid Barley Mutation Alters Seed Gene Expression

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Abstract

Barley (*Hordeum vulgare* L.) low phytic acid (*lpa*) mutants have reduced levels of seed phytate and increased concentrations of seed inorganic phosphorus. To understand how *lpa* mutations affect metabolic and developmental processes during seed growth, gene expression experiments were performed using oligonucleotide microarrays. Differential gene expression was assayed at 7 d after anthesis in developing seeds homozygous either for the *lpa* mutation M955, or for its corresponding nonmutant, wild-type allele. Homozygosity for M955 blocks phytate accumulation throughout seed development, resulting in a ~90% reduction in mature seed phytate. Consistent and substantial differential expression was observed for 38 genes (probesets) representing various cellular processes and unknown functions. The majority of differentially expressed genes showed decreased expression with a much smaller proportion of upregulated genes. No changes in expression of genes thought to be directly involved in the synthesis of phytate were observed. Reduced expression was observed for functions important to carbohydrate and cell wall metabolism, cytokinin and ethylene signaling, and transport functions. These results reflect the interconnection of phosphorus, inositol phosphate, and sugar metabolism, and signaling networks regulating these metabolic and developmental pathways. The M955 *lpa* mutation appears to affect seed development and function through carbon transport and starch synthesis and may account for yield reductions previously reported for this mutation.

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Low phytic acid (*lpa*) mutants have been identified in a number of crop species, including maize (*Zea mays* L.; Raboy et al., 2000), rice (*Oryza sativa* L.; Larson et al., 2000), soybean (*Glycine max* L. Merr.; Wilcox et al., 2000), wheat (*Triticum aestivum* L.; Guttieri et al., 2004), and barley (*Hordeum vulgare* L.; Larson et al., 1998; Raboy et al., 2001; Rasmussen and Hatzack, 1998). The *lpa* trait results from mutations that reduce the amount of phytate (*myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate, $InsP_6$, syn. phytic acid) in seeds, with increases in the amount of inorganic phosphorus (Pi) and little change to the amount of seed total phosphorus. Reduced phytate accumulation in a given mutant could be due to blocks in the synthesis or supply of the two substrates *myo*-inositol (Ins) and Pi, or blocks in their conversion to phytate, or blocks in various transport or regulatory functions important to this process. Thus, *lpa* mutations impact three metabolic pools: Ins, Pi, and phytate, each important to a number of other metabolic, signaling, and developmental pathways. In addition to providing substrate for phytate synthesis, Ins has a role in many other metabolic processes, including cell wall polysaccharides, signal

Abbreviations: DAA, days after anthesis; ELIP, early light inducible protein; His-Asp, histidine-to-aspartate; IMP, inositol monophosphatase; Ins, *myo*-inositol; *lpa*, low phytic acid; MIPS, *myo*-inositol-1-phosphate synthase; PCR, polymerase chain reaction; Pi, inorganic phosphorus; QTL, quantitative trait locus; RT–PCR, reverse transcription polymerase chain reaction; *Wt*, wild type.

transduction, and stress response (Loewus and Murthy, 2000). Phytate metabolism was hypothesized to be important to phosphorus homeostasis (Strother, 1980). The regulation of cellular Pi concentration is in turn important to normal starch synthesis, at least in part via the allostearic regulation of ADP-glucose pyrophosphorylase (Smidansky et al., 2002). Finally, phytate itself represents a major pool in Ins phosphate metabolism in all eukaryotes, important to numerous signaling and developmental processes (Shears, 2004).

Despite the importance of these metabolic pools, many *lpa* genotypes are viable and healthy. Low-phytate crops are of value for improving nutrition by making essential minerals more available and for ameliorating the effects of environmental phosphorus pollution by reducing the amount of phosphorus in animal waste (Raboy, 2001). Use of this trait in production agriculture will require the development of high-yielding, adapted *lpa* crops.

The pathway of phytate synthesis has been described previously (Raboy, 2003). It begins with the conversion of glucose 6-phosphate to *myo*-inositol 3-phosphate ($InsP_1$), catalyzed by *myo*-inositol phosphate synthase (E.C. 5.5.1.4). Ins monophosphatase (E.C.3.1.3.25) then removes the phosphate group to yield Ins. After these initial steps, the pathway to phytate may be divided into three different sections (Raboy, 2003); (i) early intermediate pathways that yield Ins trisphosphates using either phosphatidylinositol phosphates (the lipid-dependent pathway), or (ii) Ins phosphates (the lipid-independent pathway); and (iii) the late Ins phosphate pathways that convert Ins trisphosphates to phytate.

The genetic basis of the *lpa* mutants is known in several crops. The soybean LR33 *lpa* mutant has a mutation in the enzyme *myo*-inositol phosphate synthase (Hitz et al., 2002). The maize *lpa*3-1 genotype was shown to be a mutation in an inositol kinase gene (E.C. 2.7.1.64; Shi et al., 2005). The maize *lpa*2 mutant (Raboy et al., 2000) is caused by mutations in a gene encoding Ins(1,3,4)P_3 5/6-kinase (E.C. 2.7.1.X; Shi et al., 2003). *Arabidopsis* mutations in genes encoding an Ins(1,4,5)P_3 3-/6-kinase (E.C. 2.7.1.127, 2.7.1.151) and an Ins polyphosphate 2-kinase (E.C. 2.7.1.X) also result in reductions in phytate (Stevenson-Paulik et al., 2005). In all cases some phytate is still produced in seeds of viable *lpa* genotypes, indicating that there is likely some redundancy or compensatory mechanisms for de novo synthesis of phytate.

Because of the multiple pathways that utilize Ins, it could be assumed that mutations affecting phytate would also impact other products. Karner et al. (2004) showed increases in Ins, sucrose, galactinol, raffinose, and stachyose in select barley *lpa* mutants. The soybean LR33 *myo*-inositol-1-phosphate synthase (MIPS) mutant was shown to have reduction in raffinose and stachyose (Hitz et al., 2002). Additionally, certain *lpa* mutants have been shown to affect seedling emergence in some environments (Meis et al., 2003; Oltmans et al., 2005), yield (Ertl et al., 1998), and composition of structural carbohydrates (E. Souza, unpublished data). In the case of barley, effects on agronomic performance of *lpa* genotypes appear greatest in hot, moisture-restricted environments and increases in severity with the level of reduction in phytate; M955, having the greatest reduction in phytate, also has the greatest reduction in yield and seed size (Bregitzer and Raboy, 2006).

Despite the evidence of altered metabolite levels and plant and seed performance, little has been done to investigate changes in gene expression in the *lpa* mutants on a genomewide scale. Gene expression studies are valuable tools to identify pathways and genes that have altered expression due to many factors, including mutation and treatments. This study investigates the changes in gene expression in developing (7 d after anthesis) seeds of a barley *lpa* (M955) mutant with a 90% reduction in phytate compared with a nonmutant, wild-type (*Wt*) control, with the intent of studying the effect of phytate reduction on gene expression. Evidence suggests that phytate begins to accumulate early in development (Yoshida et al., 1999). In this study an early developmental stage was selected to gauge gene expression differences before large phenotypic differences, such as large differences in seed phosphorus chemistry, become evident, and before the downstream impact on gene expression of these phenotypic changes.

Materials and Methods

Plant Material

The *lpa* barley mutation, M955, was isolated following sodium azide mutagenesis of seed from the two-row barley cultivar Harrington (Dorsch et al., 2003; Harvey and Rossnagel, 1984). The genotype M955 has been described previously by Raboy et al. (2001), Dorsch et al. (2003), and Ockenden et al. (2004). Three *lpa* and three nonmutant, *Wt* $BC_2F_{2:5}$ sib lines were created with M955 as the donor parent and Harrington as the recurrent parent (Raboy, unpublished data). Phosphorus accumulation of the sib lines during development has been previously documented (Bowen et al., 2006). At 7 days after anthesis (DAA), Harrington and the $BC_2F_{2:5}$ sib lines of *lpa* and *Wt* phenotypes have less than 5μg phytate per seed. By 21 DAA, Harrington and the *Wt* $BC_2F_{2:5}$ sib lines have greater than 50 μg phyate per seed, and the *lpa* $BC_2F_{2:5}$ sib lines have phytate concentrations essentially

unchanged from 7 DAA (Bowen et al., 2006). The sib lines were grown in the field at the University of Idaho's Aberdeen Research and Extension Center, near Aberdeen, ID, in 2003 and 2004 in a randomized complete block design with two replications. Cultivation practices were the same as for other spring-sown cereals in the area (Robertson and Stark, 2003). Developing heads were removed 1 wk after anthesis (7 DAA), frozen in liquid nitrogen, and placed on dry ice until storage in an ultralow (–80°C) freezer. This material was used for gene expression analysis using microarrays and reverse transcription polymerase chain reaction (RT–PCR). Developing seed was also harvested at 14 and 21 DAA and used for RT–PCR only. Developing seeds were threshed while frozen to prevent RNA degradation.

Target Synthesis and Probe Hybridization

Seed tissue was homogenized by grinding in liquid nitrogen. Total RNA was extracted from seeds using Concert Plant RNA Reagent (Invitrogen Corp., Carlsbad, CA; catalog no. 12322012) according to manufacturer's instructions. Purified total RNA was treated with TurboDNase (Ambion, Austin, TX; catalog no. 2238) to remove contaminating genomic DNA. A 10-µg subsample of total RNA diluted to 1µg µ L^{-1} was used for probe synthesis. Probe preparation was done according to the protocol recommended by Affymetrix (Santa Clara, CA). Probe synthesis, hybridization, and scanning were done at the College of Molecular Biosciences, Washington State University, Pullman, WA. The Barley1 GeneChip (Close et al., 2004; Affymetrix, catalog no. 900515) was used as the microarray platform to analyze gene expression in the different sib lines. The array contains over 22 000 probesets derived from 350 000 barley ESTs generated by an international barley consortium. Arrays using the 2003 material as probes were hybridized and scanned in April 2004, and the 2004 arrays were done in November 2004. Two *lpa* and three *Wt* chips were probed in 2003 and two *lpa* and two *Wt* chips were probed in 2004. Raw data were deposited and can be accessed in the plant gene expression database (http://plexdb.org; verified 29 May 2007), a NCBI-related database for plant microarray data.

Normalization and Analysis

Several approaches were employed to identify differentially expressed genes. The first approach compared *lpa* and *Wt* samples from individual years, followed by comparisons between years. Signals of probe sets within individual arrays were normalized within the array and scaled to a mean signal intensity of 125 using Affymetrix's data management software. Arrays representing the *lpa* or *Wt* sib selections from each year were pooled, and a *t* test was performed to filter the probesets that were differentially expressed between the genotype treatments. The filtering parameters were a *t* test *p* value of $p < 0.1$ for comparisons of *lpa* and *Wt* chips, fold changes greater than twofold increased or decreased expression in the *lpa* compared with *Wt*, expression values of signal intensity greater than 10, and "present calls" from the Affymetrix GCOS software.

Low-phytate crops are of value for improving nutrition by making essential minerals more available and for ameliorating the effects of environmental phosphorus pollution by reducing the amount of phosphorus in animal waste. Use of this trait in production agriculture will require the development of high-yielding, adapted *lpa* crops.

"Present calls" account for specific and nonspecific binding as measured by the intensity of perfect match and mismatch array-based oligos. Filtering was done using Microsoft Excel (Redmond, WA). All probesets meeting these filtering criteria were considered differentially expressed in each individual year. After considering each year separately, the filtered probesets from each year were compared, and probesets consistent in both years were considered differentially expressed in the *lpa* line.

In an effort to leverage chip replication and year replication, a second approach was used to normalize all chips. Normalization was done using dChip software (Li and Wong, 2003; www.dchip.org). Probesets were filtered by fold change (> twofold), *t* test probability (<0.05), and signal intensity (>10). The resulting sets of probesets showing differential expression between the *lpa* and *Wt* were compared to give a group of probesets that were considered differentially expressed using both methods. This was used to reduce the number of false positives and create a manageable list of probesets for additional investigation. Similar array data comparisons and lowering significance levels have been made in fruit flies to reduce error and outline results (Dierick and

Table 1. Primer sets used for relative reverse transcription polymerase chain reaction.

Gene	F primer 5′-3′	T-ann °C†	R Primer 5′-3′	T-ann °C
Actin	TATCGCTGACCGTATGAGCAA	59	CCCTCCGATCCAGACACTGT	58
Myo-inositol-1-phosphate synthase	TGCCATACGTTGGAGACAGC	58	AGTCCTCGCAGGTGTTGTGC	60
Inositol monophosphatase	CCACCGATGACCTCACCTATG	59	AATGGTGAGGCCAATCGAGAC	60
Inositol kinase	TGGTAACTTCTTCGGCGCA	59	ACCGTGGTATTGCCTTGTCCT	59
Brittle-1	CAACGGTGATCGACCATGAAC	60	GGATTCAATTGCGCTAGGCTC	60
Sucrose synthase	CTAGGCTGGACCGTGTCAAGA	59	CACCACAGACAACCACCAGGT	59
High molecular weight early light inducible protein	TTAGTGTGGCGTCATCGCAA	60	TGTTTGTCGTCGCGGAAATA	59
Pyrophosphatase	TCGTCTTCGCGCCATTCTT	60	ACGAAAGTCTTCTGCTTGGTCA	58

†T-ann, annealing temperature.

Greenspan, 2006). The phytate synthesis pathway was the exception to this approach. Because of its relevance and biological linkage to the genotypes used in these experiments, only normalized data was used for determination of significance.

Real-Time RT–PCR

Selected genes with known function in the pathway of phytate synthesis or displaying differential expression were further assayed for expression levels using real-time RT–PCR (relative RT–PCR). RNA from three *lpa* and three *Wt* sib selections of M955 developing seeds 7, 14, and 21 DAA were diluted to equal concentrations and assayed using the RT–PCR. The specific genes assayed were MIPS (GenBank Accession O65195), inositol monophosphatase (IMP), inositol kinase (Barley1 GeneChip Contig11276), pyrophosphatase (Barley1 GeneChip Contig555_at), sucrose synthase (Barley1 GeneChip Contig823_at), ADP-glucose transport protein (Brittle1; Barley1 GeneChip Contig7528_2_at), early light inducible protein (Barley1 GeneChip Contig3430), and actin (GenBank Accession AY145451). Primers were designed using PrimerExpress (Applied Biosystems, Foster City, CA) and had annealing temperatures 59°C ± 1°C and amplified a fragment of approximately 100 bp. Primer sequences and annealing temperatures are listed in Table 1. Internal controls without reverse transcriptase were used to monitor amplification of contaminating DNA in the RNA samples. A one-step SYBR Green RT–PCR kit (Sigma cat no. QR0100, Sigma-Aldrich Chemical, St Louis, MO) was used to assay gene expression on 0.2 µg total RNA. A reaction volume of 20 µL was used. The assay was performed using an ABI Prism 7000 (Applied Biosystems). The reverse transcriptase step was at 50°C for 30 min. The PCR protocol began with an initial denaturation step of 94°C for 15 min, followed by 40 cycles of 94°C for 15 s, annealing at 57°C for 30 s, and extension at 78°C for 30 s. Amplification specificity was assessed by using a dissociation protocol following the RT–PCR cycles. Fold-change differences between M955 and *Wt* were estimated using the $\Delta\Delta^{CT}$ method using actin as the control sequence (Livak and Schmittgen, 2001).

Table 2. Number of differentially expressed probesets from Aberdeen, ID, field trial in year 2003 and year 2004, those differentially expressed in both years, number of probesets called as differentially expressed by DChip when all arrays are normalized together, and the number of probesets that were selected in both years and by the DChip normalization of probesets across all arrays.

	2003	2004	Both years†	Normalized‡	Combined§
Total probesets	210	251	65	103	38
Increased expression	60	90	8	16	2
Decreased expression	150	161	57	87	36
Unknown function	97	99	27	42	14

†2003 and 2004 probeset signals normalized within microarrays, then tested by the selection criteria: fold change (>twofold), *t* test probability (<0.05), and signal intensity (>10).

‡Data derived from normalization of all chips, removing the year effect.

§Probesets in common in comparisons between years ("Both years") and normalized data.

Results and Discussion

The barley *lpa* mutation M955 was selected for this study because of its extreme *lpa* phenotype (>90% reduction in mature seed phytate). Maize mutants with phenotypes this severe are lethal as homozygotes (Raboy et al., 2001). In contrast, M955 is viable and self-fertile and has no extreme agronomic and physiological deficiencies, apart from reduced yield (Bregitzer and Raboy, 2006; Raboy et al., 2001). Phosphorus accumulation and evidence of the *lpa* phenotype appear early in seed development. However, at the earliest time points analyzed in this study (7 DAA), no differences in total P and inorganic P were observed between M955 and *Wt*, and phytate P levels were shown to be at or below detectable limits (Bowen et al., 2006; K. Peterson and V. Raboy, personal communication). M955 has been mapped to barley chromosome 1HL. However, it is

Table 3. Fold change and *p* value for the 38 probesets common in comparing 2003, 2004, and normalized data.

		Comparisons					
		2004 material		2003 material		Normalized[§]	
Probeset	Putative function or homology	Fold change[†]	*p* value	Fold change	p-value	Fold change[‡]	*p* value
Increased expression in M955 compared to wild type							
Contig22849_at	Unknown	2.42	0.043	3.18	0.007	2.33	0.017
Contig3430_at	High molecular mass early light-inducible protein	2.19	0.048	2.77	0.007	3.29	0.029
Decreased expression in M955 compared to wild type							
Contig15743_s_at	Anion exchange 3 protein	–4.96	0.053	–2.18	0.087	–3.09	0.033
Contig6938_at	B12D protein	–5.12	0.084	–3.05	0.033	–2.60	0.001
Contig1246_at	r40c1 protein, duplicated domain structure protein	–4.57	0.082	–2.06	0.018	–2.37	0.028
Contig7875_at	Unknown	–3.86	0.092	–2.90	0.038	–3.50	0.020
Contig8993_at	CUC2, no apical meristem family protein	–4.50	0.015	–3.01	0.041	–3.58	0.004
Contig4058_s_at	Bifunctional alpha-amylase/subtilisin inhibitor	–5.28	0.037	–3.54	0.020	–3.85	0.033
Contig7528_s_at	Brittle-1 protein, chloroplast precursor	–18.32	0.032	–7.06	0.076	–8.24	0.046
Contig8947_at	Cell differentiation protein	–4.21	0.030	–2.54	0.023	–3.04	0.012
HB12G06r_at	Cell differentiation protein	–2.16	0.043	–3.57	0.072	–2.28	0.013
Contig9782_at	Chloroplast nucleoid DNA binding protein	–3.80	0.052	–2.28	0.024	–2.65	0.036
Contig17040_at	Cystatin	–3.50	0.089	–3.26	0.039	–3.55	0.013
Contig9071_at	DNA binding protein	–4.69	0.023	–2.36	0.047	–2.88	0.006
Contig4907_s_at	Ethylene-response protein	–5.04	0.017	–4.32	0.103	–5.27	0.005
Contig12093_at	Expansin-like protein	–4.36	0.094	–2.32	0.086	–2.62	0.005
Contig8334_s_at	Histidine-containing phosphotransfer protein	–5.14	0.043	–3.20	0.059	–3.64	0.029
Contig555_at	Pyrophosphatase	–7.94	0.045	–3.30	0.052	–4.15	0.004
Contig5123_at	Isoflavone reductase-like protein	–4.81	0.089	–2.34	0.030	–2.89	0.007
Contig10741_at	Nicotianamine synthase 1	–5.04	0.072	–2.37	0.011	–3.16	0.037
Contig13813_at	Nucleoid DNA-binding protein cnd41-like protein	–3.34	0.079	–2.68	0.043	–3.54	0.003
Contig2700_x_at	Putative protein	–4.09	0.083	–2.33	0.042	–2.60	0.022
Contig3692_s_at	sensory box histidine kinase/response regulator	–4.51	0.056	–2.78	0.005	–3.01	0.002
Contig823_at	Sucrose synthase 2	–5.70	0.013	–5.03	0.094	–5.61	0.031
Contig7380_at	Transcription binding factor	–4.49	0.037	–3.58	0.041	–4.10	0.008
Contig14913_at	Unknown	–10.20	0.019	–6.45	0.050	–3.95	0.037
Contig16244_s_at	Unknown	–4.11	0.060	–3.31	0.044	–3.32	0.041
Contig25559_at	Unknown	–2.72	0.033	–2.64	0.018	–3.62	0.039
Contig3747_s_at	Unknown	–4.54	0.037	–2.31	0.042	–2.93	0.026
Contig4002_s_at	Unknown	–4.75	0.039	–2.98	0.079	–4.04	0.034
Contig6702_at	Unknown	–5.34	0.017	–4.76	0.074	–4.24	0.011
Contig8303_at	Unknown	–3.66	0.100	–2.37	0.006	–2.72	0.009
Contig8304_s_at	Unknown	–5.03	0.070	–2.39	0.008	–2.64	0.026
EBed02_SQ003_G17_at	Unknown	–6.28	0.036	–5.73	0.035	–8.35	0.036
HB07K19r_x_at	Unknown	–21.25	0.006	–8.97	0.089	–9.77	0.044
HB23015r_s_at	Unknown	–6.13	0.037	–4.11	0.023	–5.15	0.033
HVSMEh0082A13r2_s_at	Unknown	–2.80	0.032	–2.16	0.022	–2.18	0.040
Contig4457_at	VsaA-like protein	–3.29	0.038	–2.43	0.018	–2.67	0.001

[†]Fold change and *t* test probability determined using Microsoft Excel.

[‡]Fold change and *t* test probability determined using dChip.

[§]Normalization values from dChip software where probesets were filtered by fold change (> twofold), *t* test probability (<0.05), and signal intensity (>10).

Table 4. Fold change and standard deviations from relative reverse transcription polymerase chain reaction results of 8 genes over 3 wk during seed development comparing M955 and wild type. Positive values indicate increased expression in M955; negative values indicate reduced expression in M955 compared with wild type.

RNA source	Myo-inositol-1-phosphate synthase (MIPS)		Inositol monophosphatase (IMP)		Inositol kinase		Early light inducible protein		Pyrophosphatase		*Brittle1*		Sucrose synthase		Actin	
	Fold change (SD) and probability that low phytic acid is significantly different by wild type															
	2003															
7 DAA†	1.51	(0.32)*	1.67	(0.41)ns‡	1.15	(0.60)ns	7.64	(1.94)***	–2.13	(0.26)***	–32.48	(1.07)***	–3.49	(1.55)ns	–1.30	(0.92)ns
14 DAA	1.47	(0.16)**ns	1.50	(0.27)ns	–2.40	(0.62)***	2.48	(0.72)*	1.06	(0.13)ns	1.29	(0.11)ns	2.02	(0.24)ns	–1.11	(0.31)ns
21 DAA	–1.41	(0.42)**ns	–1.25	(0.21)ns	–1.56	(0.51)ns	1.32	(1.00)ns	–1.56	(0.17)ns	–1.95	(0.46)*	–1.85	(0.45)ns	1.18	(0.86)ns
	2004															
7 DAA	1.89	(0.49)***	–1.72	(3.46)ns	–1.61	(0.84)*	4.22	(0.97)**	–3.35	(0.43)***	–2.25	(0.25)**	–6.05	(0.77)ns	1.69	(0.79)*
14 DAA	1.03	(0.27)ns	1.21	(0.33)ns	1.16	(0.51)ns	1.02	(1.84)ns	1.02	(0.28)ns	–1.03	(0.26)ns	1.14	(0.26)ns	–1.01	(0.21)ns
21 DAA	–1.02	(0.38)ns	1.20	(0.25)ns	–1.24	(0.50)ns	1.69	(0.98)ns	1.03	(0.31)ns	1.64	(0.20)ns	1.73	(0.11)ns	1.67	(0.28)*

*Significant at $p = 0.05$.

**Significant at $p = 0.01$.

***Significant at $p = 0.001$.

†DAA, days after anthesis.

‡ns, not significant.

not clear if it is allelic to the barley lpa3-1 mutation (Roslinsky, 2002).

Data filtering of microarray results from 2003 and 2004, followed by normalization and comparison of combined data, ultimately identified 38 probesets for which substantial (> twofold) differential expression was consistently observed (Table 2). Two showed increased expression, and 36 showed decreased expression in M955 compared with *Wt* (Table 3). The largest proportion of genes had unknown function. The large difference in the number of probesets with increased versus decreased expression is evident in all methods of analysis of the data (Table 2). A complete list of differentially expressed probesets from each year, and the normalized and combined analyses can be found in Supplemental Tables 1–4.

The data filtering approach effectively identified an initial probeset of differentially expressed genes that have interesting roles in the context of the *lpa* mutation. Probesets representing a vacuolar H^+/pyrophosphatase were identified with reduced expression in the *lpa* mutant. This protein's activity pumps protons into vacuoles, establishing an electrochemical gradient required for various transport functions. An H^+/pyrophosphatase is localized in the membranes of phytate-accumulating globoids found within one class of protein storage vacuoles (Jiang et al., 2001). In an analysis of mature seeds, Ockenden et al. (2004) found that four barley *lpa* genotypes, including M955, had altered globoid formation, particularly in the aleurone fraction of the seed. Globoids of M995 were smaller and had greater size variation in both the aleurone and scutellar cells compared with *Wt* cells. A survey of *Arabidopsis* accessions and subsequent mapping found that natural variation in phytate and Pi accumulation was likely explained by changes in transport rather than a biosynthetic pathway alteration (Bentsink et al., 2003). A quantitative trait locus (QTL) identified in that study also colocalized with a QTL for variation in seed mineral content of K, Ca, Mn, and possibly Fe (Vreugdenhil et al., 2004). Mutations in transport functions could influence the ability of a plant to store phytate.

Reduced expression of two genes involved in sugar transport and starch synthesis also was observed in M955 compared with *Wt*. Sucrose synthase expression was reduced fivefold in M955. An important function of sucrose synthase activity during seed development is to maintain the "sink strength" for carbon through catalysis of sucrose to simple sugars suitable for starch synthesis (Sturm and Tang, 1999). Karner et al. (2004) reported increased mature seed sucrose concentrations in the M955 mutant, as well as increases in Ins and galactinol. The results of the microarray analysis, while clearly significant, were not consistent with the relative RT–PCR results (Table 4). Sucrose synthase expression during the time course of the seed development was not affected by the M955 *lpa* mutation in the relative RT–PCR analysis. This ambiguity suggests that *lpa* mutation's reduction in transcript numbers for the sucrose synthase gene may be transient in early seed development but also that the effect requires further study to confirm the type of relationship between the *lpa* phenotype and sucrose synthase expression.

In contrast, both microarray and relative RT–PCR demonstrated a consistent reduction in expression of *brittle1*-like gene in early seed development (Table 3, 4). The reduced expression is transient in the seed with the *lpa* mutation having no effect at 14 and 21 DAA. Starch synthesis and transport and cell wall synthesis is mediated by ADP-glucose transport proteins encoded by genes similar to *brittle1*. This gene's product is found on the inner membranes of plastids, mainly in the endosperm. It is an ADP-glucose transport protein, activity of which provides a critical substrate for starch synthesis. Deficiency of the gene results in greatly reduced rates of starch synthesis and translocation (Johnson et al., 2003; Shannon et al., 1998). No obvious differences in endosperm structure have been reported in M955, as is evident in *brittle-1*-like mutants. However, reductions in expression of activities like *brittle-1* and sucrose synthase could, via reduced starch accumulation, contribute to reduced seed weight, test weight, and yield observed in M955, compared with their appropriate *Wt* controls (Fig. 1; Bregitzer and Raboy, 2006; Ertl et al., 1998; Raboy et al., 2000; Raboy et al., 2001). Seed weight during development was also reduced in M955 compared with *Wt* (Bowen et al., 2006).

Reduced expression was observed for genes encoding two functions that are part of the "histidine-to-aspartate" (His-Asp) phosphorelay pathway, the molecular mechanism underlying cytokinin signal transduction (Lohrmann and Harter, 2002; Sheen, 2002). In higher plants the His-Asp phosphorelay pathway has three components. Cytokinin "receptor kinases" span the plasma membrane, perceive the extracellular cytokinin signal, and in response, transfer a single phosphate group, first intramolecularly (from a histidine to an aspartate) and then intermolecularly to the system's next component, a cytoplasmic histidine-containing "phosphotransfer protein." The phosphotransfer protein then transfers the same phosphate molecule to a histidine kinase "response regulator." Upon activation (phosphorylation), the "response regulator" then functions as a transcription factor to effect changes in gene expression. Consistent and substantial reductions in expression were observed for representatives of two of these three pathway components, Contig8334_s_at and Contig3692_s_at, representing a phosphotransfer protein and a response regulator, respectively (Table 3).

Cytokinin signaling plays a central role in how plants sense and respond to nutrient status, including nitrogen, sulfur, and phosphorus status (Ferreira and Kieber, 2005). Briefly, low nutrient levels result in reduced cyotkinin signaling output, which in turn results in enhanced root growth accompanied by induction of expression of functions such as nutrient-

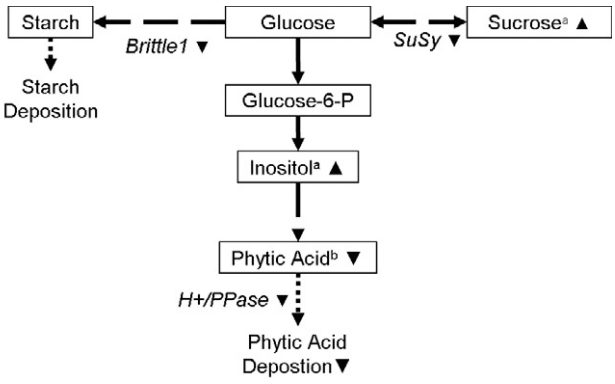

Figure 1. Relationship of inositol, phytic acid, and genes with differential expression in M955. Dashed lines indicate multiple steps and dotted lines indicate transport or non-biosynthetic reactions. (a) Metabolite levels in mature seed from Karner et al. (2004). (b) Metabolite levels in mature seed from Dorsch et al. (2003).

specific transporters, together providing for enhanced nutrient uptake. During the later phase of seed development in *lpa* mutants, seed Pi is greatly elevated compared with *Wt*. Cells of developing *lpa* seeds might respond to high Pi with high cytokinin signal output, the opposite of the results observed here. However, at 7 DAA, seed Pi levels in M955 and *Wt* are similar; the high Pi phenotype is not yet observed.

Alternatively, reduced phytate synthesis, rather than high Pi, may be the proximal cause of this altered His-Asp phosphotransfer pathway transcription. Ins phosphates, including phytate, may have a direct role in the induction of genes in response to phosphate starvation, via a role in chromatin remodeling (Shen et al., 2003; Steger et al., 2003). Stevenson-Paulik et al. (2005) demonstrated that a mutation in the *Arabidopsis Ipk1* gene, which encodes an Ins polyphosphate 2-kinase whose activity catalyses the last step in phytate synthesis, results in the inability of the plant to sense phosphate levels, in turn resulting in luxury uptake of phosphate and phosphate toxicity symptoms. Although evidence indicates that the M955 mutation impacts phytate synthesis throughout seed development, a difference in phytate concentration between M955 and *Wt* seed at 7 DAA has not yet been demonstrated (Bowen et al., 2006). A third possibility is that it is not high Pi nor reduced phytate but rather reduced expression of the protein encoded by the M955 gene that directly impacts transcription of other genes. The yeast *Ipk2* gene, which encodes an Ins polyphosphate 6-/3-kinase targeted to the nucleus, was first identified as a protein chaperone important for the functioning of a transcription regulatory complex that in turn is important to arginine metabolism and nitrogen nutrition (*Arg82*; Odom et al., 2000). The *Arg82* Ins

Table 5. Expression levels of probesets with annotated function involving inositol and phytate synthesis.

Chip designation	Description	2003		2004	
		Fold change	*t* test	Fold change	*t* test
Contig3562_at	Phosphatidylinositol-phosphatidylcholine transfer protein	–1.01	0.81	–1.16	0.36
Contig3592_at	*Myo*-inositol-1-phosphate synthase	1.05	0.89	1.02	0.92
Contig4378_s_at	Phosphatidylinositol transfer-like protein	–1.04	0.57	–1.12	0.16
Contig5912_at	Phosphatidylinositol-4-phosphate 5-kinase	1.19	0.25	1.03	0.93
Contig5913_s_at	Phosphatidylinositol-4-phosphate 5-kinase	–1.22	0.33	–1.02	0.42
Contig6304_at	Phosphatidylinositol transfer-like protein	1.06	0.85	–1.41	0.07
Contig6366_at	Inositol 2-dehydrogenase	1.13	0.27	–1.14	0.43
Contig6944_at	*Myo*-inositol-1(or 4)-monophosphatase	–1.09	0.47	–1.25	0.40
Contig7453_at	Inositol monophosphatase	–1.12	0.56	1.04	0.81
Contig8470_s_at	*n*-acetylglucosaminyl-phosphatidylinositol-like protein	1.85	0.17	–1.26	0.58
Contig8472_at	*n*-acetylglucosaminyl-phosphatidylinositol-like protein	3.77	0.01	1.33	0.37
Contig10166_at	Inositol hexaphosphate kinase	1.07	0.22	1.18	0.33
Contig10423_at	Phosphatidylinositol-4-phosphate 5-kinase	–1.17	0.23	–1.18	0.14
Contig10581_at	Phosphatidylinositol synthase	–1.02	0.92	1.03	0.92
Contig11162_at	Phosphatidylinositol 3-Kinase	–1.25	0.04	–1.16	0.03
Contig14213_at	Phosphatidylinositol-phosphatidylcholine transfer protein	–1.46	0.10	–1.59	0.02
Contig14606_at	Phosphatidylinositol-4-phosphate 5-kinase	1.06	0.79	–1.14	0.16
Contig15145_at	Phosphatidylinositol-4-phosphate 5-kinase	–1.16	0.51	–1.51	0.10
Contig15158_at	Inositol-1, 4, 5-trisphosphate 5-phosphatase	–1.41	0.39	–1.05	0.89
Contig15158_s_at	Inositol-1, 4, 5-trisphosphate 5-phosphatase	–1.43	0.09	1.19	0.41
Contig15871_at	Phosphatidylinositol kinase	–1.20	0.64	–1.16	0.15
Contig16267_at	Phosphatidylinositolglycan class N short form	1.29	0.32	1.16	0.50
Contig18940_at	Phosphatidylinositol 4-kinase	–1.09	0.59	–1.10	0.31
Contig20511_at	Inositol polyphosphate-5-phosphatase	1.28	0.76	1.52	0.64
Contig21366_at	Inositol 1,3,4-trisphosphate 5/6-kinase	–1.07	0.39	–1.16	0.47
Contig24754_at	Inositol 1,3,4-trisphosphate 5/6-kinase	–1.05	0.75	–1.01	0.95
Contig22099_at	Inositol polyphosphate-5-phosphatase	1.04	0.92	–2.49	0.19
Contig22270_at	Inositol 1,3,4-trisphosphate 5/6-kinase	–1.17	0.10	–1.06	0.74
HV_CEb0008E13r2_at	Inositol 1,3,4-trisphosphate 5/6-kinase	–1.29	0.08	–1.23	0.33
HY09G09u_at	Phosphatidylinositol-4-phosphate 5-kinase	2.17	0.02	1.30	0.20
HS09B07u_at	*n*-acetylglucosaminyl-phosphatidylinositol	–1.02	0.94	–1.01	0.95
HT01A16w_at	*Myo*-inositol-1-phosphate synthase	1.17	0.55	1.24	0.52
HW09I06u_at	Phosphatidylinositol phosphatidylcholine transfer protein	–1.91	0.14	–3.72	0.10
HV_CEb0023K20r2_at	Inositol-1, 4, 5-trisphosphate 5-phosphatase	–1.49	0.50	–1.34	0.64
HVSMEl0007A18r2_at	Inositol 2-dehydrogenase	–1.24	0.41	–1.09	0.79

phosphate kinase domain is separate from its transcription factor domain (El Alami et al., 2003). An *Arabidopsis Ipk2* homolog, *AtIpk2β*, also encodes a protein targeted to the nucleus that has separate functions both as a mediator of transcription control and as an Ins polyphosphate kinase (Xia et al., 2003). Thus, changes in gene expression observed in developing M955 seed compared with *Wt* could be due to loss of function of the M955-encoded protein as a mediator of control of transcription, rather than due to changes in phytate or Pi. The significance of the genes represented by these sequences was not recognized until later in these experiments, and therefore they were not selected for relative RT–PCR analysis. Additional research on these genes is warranted to fully understand the impact of the *lpa* phenotype on cytokinin signal transduction.

Only two probesets showed increased expression in M955. One has unknown function, and the other represents an early light inducible protein (ELIP). This protein is found in the chloroplast and is thought to play a role in light stress response (Król et al., 1999) and chlorophyll binding (Adamska et al., 1999). These two roles have no obvious relationship

to phytate synthesis and metabolism. Relative RT–PCR analysis of the ELIP expression confirmed that mRNA transcripts of this gene are at significantly higher levels in the M955 *lpa* mutant than in the comparable *Wt* line. The relative difference is greatest at 7 DAA and declines to insignificant differences between genotypes at 21 DAA (Table 4).

In the microarray analysis, none of the genes known to be involved in Ins and phytate synthesis were found to be differentially expressed between the *lpa* and *Wt* genotypes in both years, with the exception of phosphatidylinositol 3-kinase (Contig11162_at), which had a relatively minor 1.25-fold change in 2003 and a 1.16-fold change in 2004 (Table 5). Relative RT–PCR measured the expression of MIPS, IMP, and inositol kinase. Similar to the microarray analysis, IMP was not observed to have a fold change between genotypes in the relative RT–PCR analysis. However, MIPS was found to have transient increased expression (Table 4). Similarly, inositol kinase expression had transient differences between genotypes in the RT–PCR analysis with increased expression in the *lpa* phenotype at some sample times (Table 4). Based on this analysis, M955 has impacts on pathways leading to phytate synthesis, but the trends were relatively small and inconsistent in this study and certainly too small to account for the extreme *lpa* phenotype of M955, suggesting that these changes are likely a subsequent effect of the mutation on another locus rather than the causal mutations themselves.

At the beginning of the project, a working hypothesis was that there would be effects on genes specifically involved in the pathway of Ins phosphates and phytate synthesis, which would partially compensate for the reduction in phytate. These data show only limited modulation of gene expression as determined by real-time RT–PCR. In the microarray analysis, approximately 35 probesets on the Barley1 GeneChip have annotated function that involves inositol. Nearly all of these sequences had expression levels that are essentially unchanged when comparing the *lpa* and *Wt* genotypes (Table 5). Real-time RT–PCR of MIPS, IMP, and inositol kinase confirmed the fact that these genes were expressed. However, there were no consistent differences in the expression level between the *lpa* and *Wt* lines at this developmental point or 2 wk after. This finding in itself is important for understanding the nature of the *lpa* mutations, suggesting that large changes in phytate levels induced by *lpa* mutations do not appear to induce commensurate changes in the expression of known genes in the synthetic pathway for phytate synthesis.

A limitation in using gene expression data is that they provide only a glimpse into the state of the organism with respect to the gene transcripts that are being produced and do not consider sequence changes that result in alterations in post-transcription or protein structure and activity. The gene perturbed in M955 has not yet been identified, so mutations within the genes in the pathway of phytate synthesis and outside transcript regulation may be the cause of the mutation. This evidence shows that the M955 mutation has limited or no effect on the expression of these key phytate-related genes early in seed development. The small number of probesets with increased expression, compared with the majority of probesets that show decreased expression, suggests that early in development there is not directed upregulation of other genes to deal with the reductions in phytate. Gene expression experiments using tissues representing later stages of seed development may lead to additional clues into the effects of the mutation, as the phenotypic differences between mutant and *Wt* in terms of both Pi and phytate become more pronounced.

Conclusions

The regulation of growth and metabolism in plants in response to a complex array of developmental and environmental signals, varying nutrient needs and varying production and supply of sugars, is coordinated via "crosstalk" or interactions between hormone and signaling pathways (Gibson, 2003). A recent study involving one the *Arabidopsis* genome's cytokinin pathway receptor histidine kinases (Franco-Zorrilla et al., 2005) demonstrated "crosstalk" between cytokinin, sugar, and Pi-starvation signaling. Similarly, there is coordination and crosstalk between ethylene and sugar signaling pathways (Gazzarrini and McCourt, 2003). In the present study, in addition to reduced expression of two components of the His-Asp phosphorelay pathway, expression of a gene (Contig4907_s_at; Table 3) encoding an ethylene response protein was reduced in 7-DAA M955 compared with *Wt*. Therefore, the comparison of genomewide transcription conducted here between 7-DAA M955 and *Wt* seeds is consistent with crosstalk, or coordinated interaction, between sugar, cytokinin, ethylene, and Ins phosphate signaling pathways in developing barley seeds. The main genetic support for this conclusion comes from the microarray analysis. Relative RT–PCR experiments were not conducted to validate the crosstalk hypothesis. Given the sometimes inconsistent results between our microarray and RT–PCR results relative to the sucrose synthase probesets, the observed coordinated interaction modulated

by M955 should be considered a significant initial observation to be confirmed in future investigations.

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